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Survival of Chlorophyllous and Nonchlorophyllous Fern Spores Through Exposure to Liquid Nitrogen

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ABSTRACT.—Thirty-three species of ferns with nonchlorophyllous spores and five species with chlorophyllous spores were studied in regard to their ability to survive exposure to liquid nitrogen (LN). Air dried spores showed no inhibition of germination after LN exposure when planted on soil or growth medium. Spores of three species that were stored for 75 months either at 4°C, −20°C, or in LN showed no decrease in viability over that time, and spores of four other species were maintained successfully for 52 months in LN. Fresh chlorophyllous spores that were air dried, dried over silica gel, or prepared with the encapsulation dehydration procedure also showed good survival through desiccation and LN exposure. Spores of *Osmunda regalis* germinated well after 18 months of LN storage. These results indicate that both nonchlorophyllous and chlorophyllous spores are candidates for long-term germplasm storage at low temperatures, including storage in LN.

Ex situ preservation of spores, particularly of rare species from threatened habitats, can be an important supplement to the maintenance of pteridophyte species in the wild. Many species of homosporous ferns produce nonchlorophyllous spores that can be stored for relatively long periods of time, although they can eventually lose viability in storage (Beri and Bir, 1993; Camloh, 1999). It has also been shown that hydrated spores of some of these species can remain viable in the soil spore bank for a number of years, and moist storage of spores ex situ has also been effective (Lindsay *et al.*, 1992; Dyer, 1994). Other species of ferns and fern allies produce chlorophyllous spores, which generally have limited viability, even though they may survive desiccation (Lloyd and Klekowski, 1970).

Low temperature storage has been shown to improve the longevity of dry seeds (Dickie *et al.*, 1990), and lower temperatures improved the survival of spores of the tree fern, *Cyathea delgadii* during two years of storage (Simabukuro *et al.*, 1998). Cryopreservation, or storage at −196°C in liquid nitrogen (LN), has the potential for maintaining viability in living tissues over long periods of time. Many seeds can survive direct exposure to LN (Stanwood, 1985; Pence, 1991), and spores of the endangered tree fern, *Cyathea spinulosa* have been successfully germinated after cryopreservation (Agrawal *et al.*, 1993).

In order to examine the possibility of extending cryopreservation to other species of ferns, spores of several nonendangered chlorophyllous and nonchlorophyllous species were tested for their ability to withstand LN exposure, and storage in LN was compared with other low temperature storage methods. Results from tests with these common species should provide direction for

future work with rare or endangered germplasm, which is often in limited supply.

Materials and Methods

Spores were obtained from a variety of sources, including from the American Fern Society Spore Exchange and from fronds collected in northwest Trinidad, at the Krohn Conservatory (Cincinnati) and at the Cincinnati Zoo and Botanical Garden.

When fronds were collected, they were air dried for several days under ambient conditions in the laboratory in paper envelopes, with spores collected from the envelopes and by scraping the fronds. Samples of the spores were then transferred to 2 ml polypropylene cryovials and immersed directly into LN. After 1 hr, the vials were removed and placed on the benchtop to warm at ambient temperature for 20 min.

LN exposed and control nonchlorophyllous spores of 33 species were tested for germination using one of the two following methods.

1) Spores of some species were sown on moist sterile soil (Metro Mix 250) in Magenta boxes or baby food jars with Magenta caps, and incubated at 26°C under CoolWhite fluorescent lights in a 16/8 hr light/dark cycle.

2) Spores of other species were germinated in vitro. Spores were wrapped in a small package made from Whatman No. 1 filter paper, and surface sterilized in a 1:20 dilution of commercial sodium hypochlorite for 5 minutes, followed by two rinses in sterile, ultra-pure water. The spores were then blotted onto medium consisting of half-strength Linsmaier and Skoog (1965) (LS) medium with 1.5% sucrose and 0.22% Phytagel (Sigma Chemical Co.), in 60 × 15 mm disposable petri dishes, approximately 15 ml/dish.

For longer storage, nonchlorophyllous spores of three species were placed in multiple cryovials and stored at three temperatures: LN (−196°C), −20°C, and 4°C. The cryovials stored at −20°C and 4°C were placed inside 20 ml borosilicate scintillation vials with plastic screw caps, containing 2–2.3 g of silica gel. Spores from the three storage temperatures were sampled at 7, 13, 34, and 75 months by germinating on soil, as described above. Spores from four species collected in Trinidad were also cryostored in LN.

Chlorophyllous spores of five species were also tested. These were obtained either from the Spore Exchange or from plants growing at the Cincinnati Zoo and Botanical Garden. In most cases these were air dried, as for the nonchlorophyllous spores.

However, *Osmunda regalis* spores which were collected from plants grown at the Cincinnati Zoo and Botanical Garden were tested using two other procedures. One portion of the spores was dried in a desiccator with silica gel overnight, placed in a cryovial, and frozen and thawed as described above. The spores were then placed in a 100% humidity chamber for 3 hours, and surface sterilized and germinated in vitro as described above. A second portion of the spores was first surface sterilized and then encapsulated in alginate according to the procedure of Fabre and Dereuddre (1990). The spores were

blotted into a 3% solution of low viscosity alginic acid (Sigma Chemical Co.) and the spore suspension was added dropwise to a 100 mM solution of calcium chloride, where the drops gelled and formed alginate beads containing the spores. After 20 min the beads were transferred to a solution of LS medium containing .75M sucrose and incubated on a gyratoray shaker, 125 rpm, for 18 hours. They were then dried on filter paper under the air flow of a laminar flow hood for 4 hours, transferred to 2 ml polypropylene cryovials, immersed directly into LN, and maintained there for 1 hour. The beads with spores were warmed at room temperature for 20 minutes and then transferred to half-strength LS medium for rehydration and growth. Some dried beads were also put into long-term LN storage, and a sample was removed after 18 months for growth and evaluation.

Only a qualitative evaluation of spore germination was made, both on soil and in vitro, although an attempt was made to maintain approximately equal amounts of control and LN exposed spores. Positive spore germination was recorded if any germination was observed. With the nonchlorophyllous spores, no great differences in the rate of germination were observed between control and frozen spores of any species, as determined by gross observation. With chlorophyllous spores, a distinction was made between the germination of many spores and the germination of one or only a few spores, as indicated in Table 3.

Results

The germination of air-dried nonchlorophylous spores exposed to LN was equivalent to that of nonexposed spores for the species tested (Table 1). Germination rates varied between species, as determined by visual examination of the germination of similar amounts of spores, but there were no obvious differences between LN exposed and control spores of the same species. In the case of three species, there was no germination from either control or LN exposed spores.

There was also no apparent difference between the germination of spores of three species stored at 4°C, −20°C, or in LN, after more than six years of storage (Table 2). Four species from Trinidad (*Adiantum tenerum, Cyclopeltis semicordata, Macrothelyptris torresiana,* and *Tectaria incisa)* were also placed into long-term LN storage and germinated well after 52 months of storage.

Chlorophyllous spores of five species were also tested (Table 3). There was no germination observed for *Blechnum nudum, Matteuccia struthiopteris,* or *Osmunda cinnamomea* spores, that were obtained after storage at the Spore Exchange for several months. With *Onoclea sensibilis* spores obtained from the Spore Exchange, only one spore germinated in the nonexposed samples and none in the LN exposed sample, suggesting a low viability of the spores overall. However, when fresh spores of *O. sensibilis* were collected locally and tested, there was germination of the dried spores, both with and without LN exposure. Similarly, when spores of *Osmunda regalis* were obtained from a nonlocal source after about a year of dry storage, there was very little germi-

TABLE 1. Survival of 33 species with nonchlorophyllous spores through LN exposure after air drying; germination = +; no germination observed = −; contaminated, germination could not be observed = C. Spore sources: K = Krohn Conservatory; SE = Spore Exchange; T = Trinidad collection; Z = collection at the Cincinnati Zoo and Botanical Garden.

Species	Source	Germination	
		Control	LN expose
Germinated in vitro			
Adiantum caudatum L.	K	C	+
Adiantum tenerum Sw.	K	+	+
Adiantum trapeziforme L.	K	+	+
Cibotium glaucum (Sm.) Hook & Arn.	K	+	+
Cyrtomium falcatum (L.f.) C. Presl	K	+	+
Davallia fejeensis Hook.	K	+	+
Drynaria quercifolia (L.) J. Sm.	K	+	+
Phlebodium aureum (L.) J. Sm.	K	+	+
Germinated on soil			
Adiantum tenerum Sw.	T	+	+
Adiantum tetraphyllum Humb. & Bonpl. ex Willd.	T	+	+
Asplenium ruta-muraria L.	SE	+	+
Asplenium platyneuron (L.) Britton, Sterns, & Poggenb.	Z	+	+
Athyrium filix-femina (L.) Roth	Z	+	+
Athyrium thelypteroides (Michx.) Desv.	Z	+	+
Bolbitis sp.	K	−	−
Cibotium schiedei Schltdl. & Cham.	K	+	+
Cyathea arborea (L.) Sm.	SE	+	+
Cyclopeltis semicordata (Sw.) J. Sm.	T	+	+
Cyrtomium falcatum (L.f.) C. Presl.	Z, K	+	+
Cyrtomium fortunei John Sm.	Z	+	+
Dryopteris carthusiana (Vill.) H. P. Fuchs	Z	+	+
Dryopteris celsa (W. Palmer) Small	SE	+	+
Dryopteris clintoniana (D.C. Eaton) Dowell	SE	+	+
Dryopteris goldiana (Hook.) A. Gray	Z	+	+
Dryopteris marginalis (L.) A. Gray	Z	+	+
Macrothelyptris torresiana (Gaud.) Ching	K, T	+	+
Nephrolepis sp.	Z	+	+
Phegopteris connectilis (Michaux) Watt	SE	−	−
Polystichum acrostichoides (Michx.) Schott	Z	+	+
Polystichum aculeatum (L.) Roth	Z	+	+
Polystichum braunii (Spenn.) Fée	Z	+	+
Polystichum tsus-simense (Hook.) J. Sm.	K	+	+
Pteris sp.	K	+	+
Rumohra adiantiformis (G. Forst.) Ching	K	−	−
Tectaria incisa Cav.	T	+	+

nation with or without LN exposure. However, when locally collected spores were processed immediately after collection, there was good survival through drying and LN exposure, using either drying over silica gel or the encapsulation dehydration procedure. In addition, fresh spores of *O. regalis* that were cryostored using the encapsulation dehydration procedure retained good viability after 18 months of LN storage (Table 3).

TABLE 2. Survival of nonchlorophyllous spores of three species for up to 75 months at three storage temperatures.

Species	Storage time (months)	Storage temperatures		
		4°C	−20°C	−196°C
Pteris sp.	7	+	+	+
	13	+	+	+
	34	+	+	+
	75	+	+	+
Cyrtomium falcatum	7	+	+	+
	13	+	+	+
	34	+	+	+
	75	+	+	+
Polystichium tsus-sinense	7	+	+	+
	13	+	+	+
	34	+	+	C
	75	+	+	+

DISCUSSION

These results demonstrate that dried spores from a number of species of ferns can survive exposure to liquid nitrogen. In addition, dry, nonchlorophyllous spores of four species survived LN storage for 52 months, and three other species survived well for over 75 months when kept at either 4°C, −20°C, or −196°C (in LN). Chlorophyllous spores of *Osmunda regalis* maintained viability in LN for at least 18 months.

It is known that nonchlorophyllous spores of many species can be maintained in the dry state at room temperature for a number of years, or even decades (e.g., Windham *et al.*, 1986). Viability does decrease over time, and this loss has been correlated with a loss of protein, sugar, and amino acids in *Pteris vittata* (Beri and Bir, 1993). Hydrated storage has also been explored and has been shown to maintain viability longer than dry storage with spores of several species (Lindsay *et al.*, 1992). Spores of *Cryptogramma crispa* showed a decreased rate of germination when subjected to freezing at −18°C, although the degree of moisture in the spores was not determined (Pangua *et al.*, 1999).

Nonchlorophyllous spores are similar to orthodox seeds, which can be dried and stored for a number of years. The longevity of orthodox seeds is extended proportionately with a decrease in the storage temperature, and such seeds have traditionally been stored at 4°C or −20°C. Dried orthodox seeds are also generally tolerant of LN storage, which may extend longevity significantly (Stanwood, 1985; Pence, 1991). Nonchlorophyllous spores appear to have a similar tolerance for cryostorage, and, in the case of three species, there was no apparent difference in the germination of spores stored at 4°C, −20°C, or in LN for up to six years. More detailed studies are needed to determine if there are differences in the rate or the quality of germination after longer storage times, but all three temperatures should extend the storage life of nonchlorophyllous spores well beyond the mean of 3 years at room temperature, determined by Lloyd and Klekowski (1970).

TABLE 3. Survival of spores of five chlorophyllous species through LN exposure, prepared either with air drying, drying under the air flow of the laminar flow hood or with the encapsulation/pretreatment/dehydration procedure (Fabre and Dereuddre, 1990). Spore sources: SE = Spore Exchange; Z = collection at the Cincinnati Zoo and Botanical Garden. Germination of many spores = +; germination of one or only a few spores = (+); no germination observed = −.

Species	Source	Treatment	Time in LN	Survival
Blechnum nudum (Labill.) Matt ex Lerss.	SE	Air drying	0 hr	
			1	−
Matteuccia struthiopteris (L.) Tod.	SE	Air drying	0	−
			1	−
Onoclea sensibilis L.	SE	Air drying	0	(+)
			1	−
	Z	Air drying	0	+
			1	+
Osmunda cinnamomea L.	SE	Air drying	0	−
			1	−
Osmunda regalis L.	SE	Air drying	0	−
			1	(+)
	SE	Air drying	0	(+)
			1	(+)
	SE	Air drying	0	−
			1	−
	Z	Drying over silica gel	0	+
			1	+
	Z	Encapsulation/dehydration	0	+
			1	+
			0	+
			1	+
			18 mos	+

In contrast with nonchlorophyllous spores, chlorophyllous spores germinate rapidly, within an average of two days, and have a mean storage life of only 48 days (Lloyd and Klekowski, 1970), although there is evidence that in the moist soil spore bank they can survive at least 10 months (Dyer and Lindsay, 1992). No more than 5% viability was reported for *Osmunda regalis* and *O. claytoniana* spores maintained for 3.5 years at 4°C (Stokey, 1951). The limited viability of dry spores of *Equisetum hyemale* has been shown to result from damage to photosynthetic function that occurs within two weeks of storage (Lebkuecher, 1997), although their longevity has been increased by storage at 4°C and at −70°C (Lloyd and Klekowski, 1970; Whittier, 1996). Of the chlorophyllous species studied here, spores that had been stored for a year had lost most or all viability by the time of testing, whereas fresh spores were viable and maintained viability through desiccation and LN storage. In the case of *Osmunda regalis* spores, viability was maintained for at least 18 months in LN storage, and it is likely that this may extend for even longer periods of time. Freeze storage of dried chlorophyllous spores might be used by spore banks to maintain viability in these species over longer periods of time, al-

though further research is needed to determine whether conventional freezing temperatures are equally as effective as storage in LN.

Chlorophyllous spores that can tolerate desiccation but have limited viability under natural conditions resemble seeds classified as sub-orthodox (Bonner, 1986). Seeds of species of *Populus* and *Salix*, for example, can tolerate drying, but are generally short-lived either as dried or hydrated seeds. However, when they are dry, they can also tolerate exposure to freezing temperatures (−20°C and LN), and when stored under these conditions their longevity is increased compared with storage at 4°C (Pence, 1996, and unpublished). LN storage should offer a similar advantage for the long-term storage of the generally short-lived chlorophyllous spores.

The results of these studies suggest that low temperature storage, including cryopreservation, is an option for the storage of both nonchlorophyllous and chlorophyllous fern spores. Although each species is unique, these results with nonendangered species provide an indication that there is a high probability of success with spores from a variety of species, including rare or endangered species, for which spores may be in limited supply. Low temperature spore banking could be used to supplement traditional spore storage, as well as sporophyte collections, soil spore banks (Dyer, 1994), and LN storage of fern gametophytes (Pence, 2000) for the maintenance of rare or endangered species. For the long-term ex situ preservation of rare or endangered germplasm, low temperature storage, including cryopreservation, can be used as one tool in an integrated approach to fern species preservation.

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